CCL2, Galectin-3, and SMRP Combination Improves the Diagnosis of Mesothelioma in Pleural Effusions

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Introduction: Malignant pleural mesothelioma (MPM) is a highly aggressive tumor with poor prognosis. One major challenge for this disease is the development of new, early, and highly reliable diagnostic markers. The aim of this study was to compare the diagnostic value of the chemokine chemokine (C-C motif) ligand 2 (CCL2), galectin-3, and the secretory leukocyte peptidase inhibitor (SLPI) with soluble mesothelin-related peptides (SMRP), and to evaluate the diagnostic performance of marker combinations.

Methods: The levels of the different markers were measured by enzyme-linked immunosorbent assay in pleural fluids from patients with MPM (n = 61), adenocarcinomas (ADCA, n = 25), or with benign pleural effusions (BPE, n = 15).

Results: SMRP, SLPI, and CCL2 concentrations were significantly higher in pleural effusions from mesothelioma patients. Conversely, galectin-3 levels seemed to be elevated in patients with pulmonary ADCA. Receiver operating characteristic curve analysis revealed that SMRP (area under the curve [AUC] = 0.9059), CCL2 (AUC = 0.7912), galectin-3 (AUC = 0.7584), and SLPI (AUC = 0.7219) were potentially interesting biomarkers for the differentiation of MPM patients from those with BPE or ADCA. Of interest, we showed that the combination of SMRP/CCL2/galectin-3 greatly improved MPM diagnosis (AUC = 0.9680), when compared with SMRP alone.

Conclusion: The combination of SMRP/CCL2/galectin-3 seems to represent a promising panel of biomarkers for the reliable diagnosis of MPM in pleural fluids.

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Malignant pleural mesothelioma (MPM) is a rare cancer, usually associated with asbestos exposure. As a consequence of the widespread use of asbestos over the past century, and the long latency period between asbestos exposure and tumor development, the worldwide incidence of MPM is expected to continue to rise substantially over the next 2 decades.[1,2]

The diagnosis of MPM is difficult because (1) the disease may arise in patients up to 40 years after asbestos exposure, (2) the clinical and imaging signs of this cancer are rather nonspecific and may appear late, and (3) a definitive diagnosis, which relies on histology, can sometimes be very difficult to achieve.[3] It can also be difficult to distinguish MPM from benign pleural effusions (BPE)[4] or from other cancers, notably adenocarcinoma (ADCA).[5,6] In some cases, it is impossible to obtain a definitive diagnosis even after histological analysis of pleural biopsies or because tumor tissue was not available from frail patients. To date, no single marker or panel of soluble (dedicated) biomarkers has been established to obtain a clear diagnosis of MPM.[7] Such a marker or combination of markers could be very helpful for clinicians for an earlier diagnosis of mesothelioma, and perhaps for the management of the disease (disease monitoring, prediction of response to treatment, and prognostic evaluation).

Soluble markers are already used routinely in the diagnosis and/or tumor monitoring of several types of cancer.[8,9] Soluble markers, such as osteopontin[10] or soluble mesothelin-related peptides (SMRP)[11–15] have recently been identified as potentially useful in the evaluation of mesothelioma. The potential diagnostic value of SMRP in MPM was also supported by our study showing that SMRP results from an aberrant alternative messenger RNA splicing and metalloprotease shedding of mesothelin in mesothelioma cells.[16] However, SMRP is not secreted by some MPM, in particular the sarcomatoid mesothelioma subtype, which strongly limits its value in clinical practice. The identification and validation of new soluble markers are, thus, urgently needed to improve the noninvasive diagnosis of MPM. Because pleural effusion analysis is very often the first diagnostic step in patients

suspected of MPM, we focused on the assessment of soluble biomarkers in pleural fluids.

The aim of this study was to compare and combine the diagnostic value for MPM of previously and newly identified soluble markers. In a previous study, we identified the chemokine, chemokine (C-C motif) ligand 2 (CCL2), and galectin-3 (LGALS3) as new, potential soluble markers for MPM diagnosis notably to differentiate MPM from lung adenocarcinoma.[17] In the present study, levels of CCL2, galectin-3, SMRP, and secretory leukocyte protease inhibitor (SLPI) in pleural effusions from patients with suspected MPM or ADCA were compared. In addition, the expression of these soluble markers was also determined in the supernatants of cell lines (17 MPM and ADCA cell lines) that we established from pleural fluids. Individual- or combined-expression analyses of all soluble markers were performed to determine their abilities to obtain an accurate diagnosis of MPM.

PATIENTS AND METHODS

Patients

Pleural-effusion samples from patients consulting for chest pain or shortness of breath with a suspicion of MPM, or with a recent diagnosis of MPM, were aseptically collected by thoracocentesis at the Laënnec Hospital (St.-Herblain, France) between 1998 and 2010. Samples were centrifuged at 1000 *g* in a Heraeus Multifuge for 20 minutes at +4°C and supernatants were aliquoted and stored at −80°C. Diagnoses were established by both fluid cytology and immunohistochemical staining of pleural biopsies performed by our pathology department, Laënnec Hospital (St.-Herblain, France), then externally confirmed by Mesopath, the French panel of pathology experts for the diagnosis of mesothelioma, dividing the patients into three groups (Table 1): 61 patients with MPM, 25 patients with metastasis from ADCA, and 15 patients with benign pleural effusions (BPE). All recruited patients had received no prior anticancer therapy and gave signed, informed consent.

Enzyme-Linked Immunosorbent Assays

Pleural effusions were centrifuged and aliquots of pleural fluids were stored at −80°C until tested. Culture supernatants were collected as follows: 2×10^6 cells were plated by well in 6-well plates. After 24 hours, three rinses were performed with complete, fetal calf serum (FCS)-free Roswell Park Memorial Institute 1640 medium. Two milliliters of complete Roswell Park Memorial Institute 1640 medium containing 2% FCS were added to each well and culture supernatants were collected 24 hours later, centrifuged at 1000 *g* for 10 minutes, aliquoted and stored at −80°C. SMRP, SLPI, CCL2, and galectin-3 titrations were performed, respectively, with the MESOMARK immunoassay kit (CIS Bio International, Gif sur Yvette, France), the Human SLPI Assay Kit Quantikine (R&D Systems), the Human MCP-1 enzyme-linked immunosorbent assay (ELISA) kit (PromoKine), and the Human Galectin-3 ELISA kit (PromoKine) following the manufacturers' recommendations.

TABLE 1. Description of Groups and Demographic Characteristics of Recruited Patients

	BPE	MPM	ADCA
Description	15	49 epithelioid 4 mixed 4 sarcomatoid 4 unspecified	10 lung 9 others 6 unspecified
Age, y (mean ± SD)	68.0 ± 15.7	67.4 ± 18.1	67.4 ± 17.9
Male sex, (%)	86.6	83.6	32.0
Confirmed asbestos exposure (%)	26.6	44.2	16.0

BPE, benign pleural effusion; MPM, malignant pleural mesothelioma; ADCA, adenocarcinoma.

Statistical Analysis

Comparisons of each population distribution were carried out using the nonparametric Kruskal–Wallis test followed by the Dunn's post test. Biomarkers yielding p values<0.05 in the univariate analyses were entered into a multiple logistic regression model. The final model included the biomarkers independently associated with the presence of MPM. Performances of the models were assessed by the Hosmer−Lemeshow statistical test and the measurement of the areas under receiver operating characteristic (ROC) curves. The best theoretical cutoffs were calculated by minimizing the distance between the point with specificity = 1 and sensitivity = 1 and the points on the ROC curves. Analyses were performed with *R* statistical software and GraphPad Prism (Prism 5 for Windows).

RESULTS

Determination of SMRP and SLPI Levels in Pleural Fluids

According to cytological and histological diagnoses, we established a collection of 61 pleural fluid samples from patients with MPM (49 epitheloid mesothelioma [EM], four mixed mesothelioma [MM], four sarcomatoid mesothelioma [SM] and four unspecified), 25 samples from patients with ADCA metastasis and 15 samples from patients with BPE (Table 1). We initially measured SMRP levels in pleural fluids by ELISA. As shown in Figure 1*A*, SMRP was present at higher levels in pleural effusions from patients with MPM (median, 33.80 nM) than those from patients with ADCA metastasis (median, 3.40 nM) or BPE (median, 2.50 nM), with $p < 0.001$ for all comparisons. Areas under ROC were 0.902 ± 0.032 and 0.912 ± 0.035 to differentiate MPM from ADCA and MPM from BPE, respectively (Table 2). Specificities and sensitivities obtained were 81.97% and 84.00% for a cutoff of 11.45 nM to differentiate MPM from ADCA, and 75.41% and 93.33% with a cutoff of 14.60 nM to differentiate MPM from BPE (Table 3).

In a previous study,[17] we identified SLPI as a potential marker for MPM. To evaluate the MPM diagnostic value of SLPI, we measured its level in our collection of pleural fluids using ELISA. SLPI concentrations were higher in pleural fluids from patients with MPM (median, 228.20 ng/ml) than those with ADCA (median, 101.50 ng/ml) or BPE (median, 91.10 ng/ml) (Fig. 1*B*). The areas under ROC curves were 0.730 ± 0.054 and 0.706 ± 0.070 to differentiate MPM from ADCA and BPE, respectively (Table 2). A specificity of 70.49% and a sensitivity of 80.00% were obtained to differentiate MPM

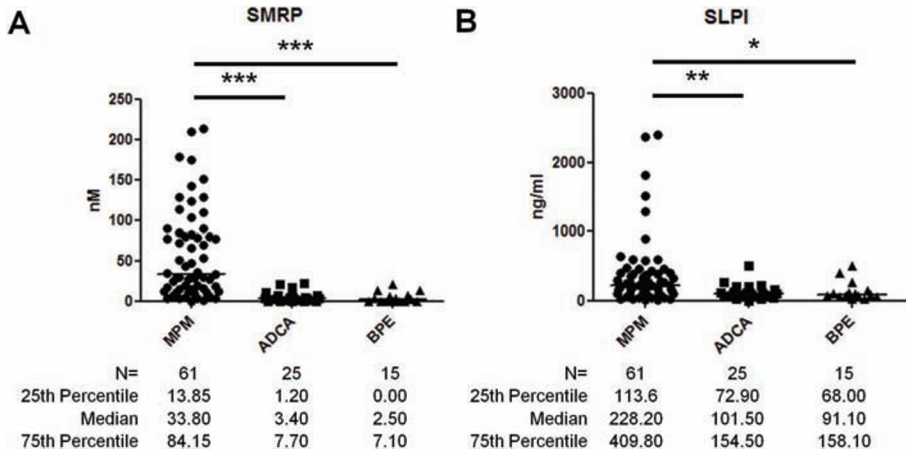

FIGURE 1. Measurements and diagnostic value of soluble mesothelin-related peptides (SMRP) and secretory leukocyte peptidase inhibitor (SLPI) in pleural fluids of patients. Pleural fluid SMRP (*A*) and SLPI (*B*) values in malignant pleural mesothelioma patients compared with adenocarcinoma and benign pleural effusion patients. Horizontal lines, median values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

from ADCA or BPE, with cutoffs of 168.20 ng/ml and 163.60 ng/ml, respectively (Table 3).

A major limit for the use of SMRP in diagnosing MPM is the lack of sensitivity of this marker, and especially in the nondetection of the sarcomatoid histological subtype. To determine whether our new soluble markers could improve MPM diagnosis, we analyzed the expression of CCL2, galectin-3, and SLPI in each subtype of cancer (Fig. 2). Pleural fluid SMRP, CCL2, and galectin-3 levels differed among patients with mesothelioma (epithelioid [EM], sarcomatoid [SM], and mixed [MM] subtypes), ADCA (lung and other origins), and BPE ($p < 0.0001$). The difference in SLPI levels among subgroups of patients was also found to be significant, but to a lesser extent ($p = 0.0052$). SMRP levels were significantly higher in EM (median: 48.30 nM) than in ADCA (pulmonary ADCA median, 2.80 nM and other ADCA median, 3.40 nM) and BPE patients (median, 2.50 nM) (Fig. 2*A*). Whereas SMRP levels seemed to be higher in EM patients than in SM and MM subgroups, no statistically significant variation between these subgroups of MPM patients was observed. CCL2 levels were significantly higher in EM and, interestingly, in SM patients (median, 2.82 ng/ml and 16.73 ng/l, respectively) than in other ADCA patients (median, 0.80 ng/ml). CCL2 levels were also higher in EM (median, 16.73 ng/ml) than BPE patients (median, 1.47 ng/ml) (Fig. 2*B*). By contrast, pulmonary ADCA patients presented significantly higher levels of galectin-3 (median, 67.33 ng/ml) in pleural fluids than did all subtypes of MPM (EM median, 11.10 ng/ml, $p < 0.0001$; SM median, 11.65 ng/ml, $p < 0.05$; and MM median, 8.79 ng/ml, $p < 0.05$) (Fig. 2*C*). No significant differences in the level of SLPI were observed among all patient subgroups (Fig. 2*D*).

TABLE 2. ROC Curve Data for Ability of SMRP and SLPI to Differentiate MPM from ADCA or BPE

Marker	AUC	95% Confidence Interval	SE	p
SMRP MPM vs. ADCA	0.902	0.8385–0.9655	0.03239	<0.0001
SMRP MPM vs. BPE	0.912	0.8433–0.9818	0.03533	<0.0001
SLPI MPM vs. ADCA	0.730	0.6250–0.8365	0.05395	0.0005
SLPI MPM vs. BPE	0.706	0.5670–0.8450	0,07090	0.013

ROC, receiver operating characteristic; SMRP, soluble mesothelin-related peptides; SLPI, secretory leukocyte peptidase inhibitor; BPE, benign pleural effusion; MPM, malignant pleural mesothelioma; ADCA, adenocarcinoma; AUC, area under the curve.

TABLE 3. Theoretical Best Cut-off Values to Differentiate MPM from ADCA or BPE

Marker	Cutoff	Specificity %	Sensitivity %
SMRP MPM vs. ADCA	11.45 nM	81.97	84.00
SMRP MPM vs. BPE	14.60 nM	75.41	93.33
SLPI MPM vs. ADCA	168.20 ng/ml	70.49	80.00
SLPI MPM vs. BPE	163.60 ng/ml	70.49	80.00

MPM, malignant pleural mesothelioma; ADCA, adenocarcinoma; BPE, benign pleural effusion; SMRP, soluble mesothelin-related peptides; SLPI, secretory leukocyte peptidase inhibitor.

Measurement of Soluble Markers in Cell-Culture Supernatants

Over several years we have developed a collection of MPM and ADCA cell lines established from pleural fluids of patients. Using this collection, we performed a transcriptomic study to compare MPM and ADCA cell lines, allowing the identification of new soluble markers.[17] To confirm these results, we measured the expression of CCL2, galectin-3, SLPI, and SMRP in cell-culture supernatants by ELISA (Fig. 3). SMRP secretion was higher in MPM than in ADCA cell-culture supernatants (22.54 nM/10^6 cells/24 hours and 10.68 nM/10^6 cells/24 hours [median values], respectively) (Fig. 3*A*). Likewise, CCL2 levels were higher in the supernatants of MPM than in ADCA cell cultures (13.03 ng/ml/10^6 cells/24 hours and

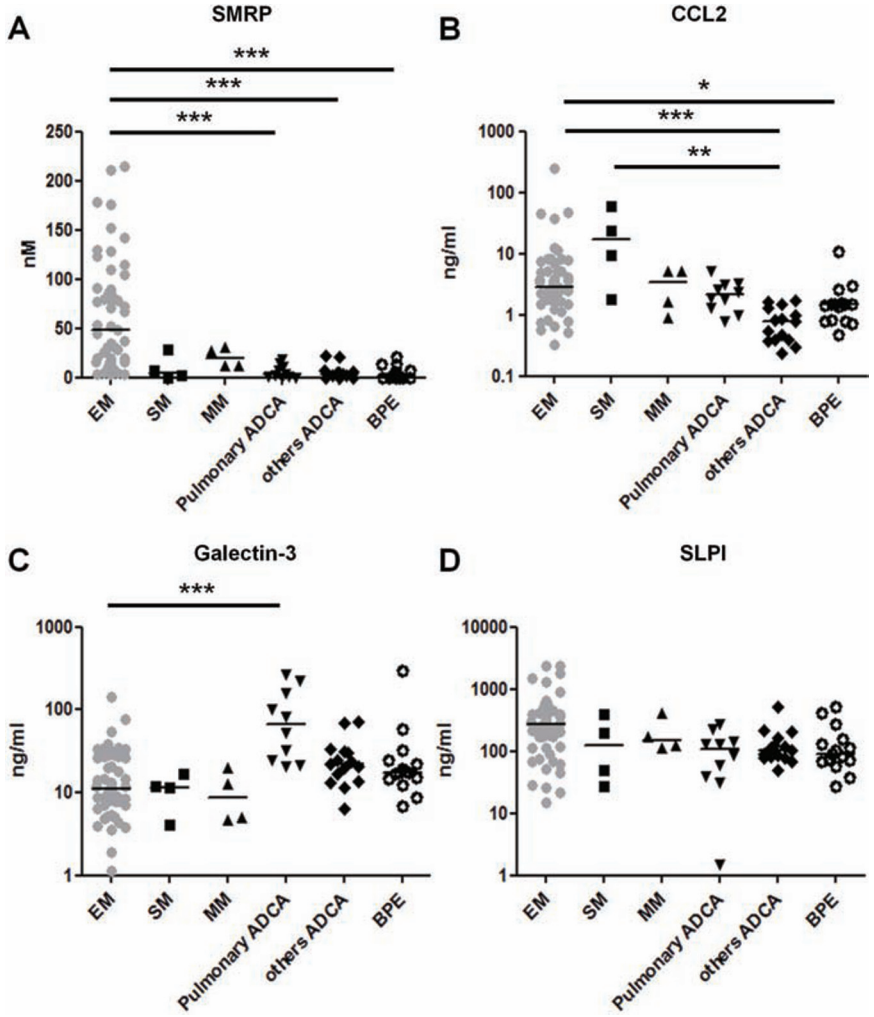

FIGURE 2. Pleural fluid levels of SMRP, CCL2, galectin-3, and SLPI in the subgroups studied. Pleural fluid SMRP (*A*), CCL2 (*B*), galectin-3 (*C*), and SLPI (*D*) values in patients with epithelioid MPM (EM), sarcomatoid MPM (SM), mixed MPM (MM), pulmonary adenocarcinoma, other ADCA (ovarian, breast and unspecified) and BPE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. SMRP, soluble mesothelin-related peptides; SLPI, secretory leukocyte peptidase inhibitor; MPM, malignant pleural mesothelioma; EM, epithelioid mesothelioma; SM, sarcomatoid mesothelioma; MM, mixed mesothelioma; ADCA, adenocarcinoma; BPE, benign pleural effusion.

2.41 ng/ml/10^6 cells/24 hours (median values), respectively) (Fig. 3*B*). Conversely, ADCA cells secreted higher levels of galectin-3 than did MPM cells (4.20 ng/ml/10^6 cells/24 hours and 1.87 ng/ml/10^6 cells/24 hours [median values], respectively) (Fig. 3*C*). Finally, SLPI levels were higher in MPM than in ADCA cell-culture supernatants (1114 pg/ml/10^6 cells/24 hours and 39.27 pg/ml/10^6 cells/24 hours [median values], respectively) (Fig. 3*D*). However, because of the spread of the values and the number of ADCA cell lines analyzed, the results observed were not statistically significant using the Mann-Whitney test.

Diagnostic Value of SMRP, SLPI, CCL2, and Galectin-3, Alone or in Combination

Figure 4*A* shows that SMRP remains the best soluble marker to differentiate MPM from ADCA and BPE (area under the curve [AUC] = 0.9059 ± 0.0283), as compared with CCL2 (AUC = 0.7912 ± 0.0454), galectin-3 (AUC = 0.7584 ± 0.0475), and SLPI (AUC = 0.7219 ± 0.0516) (Table 4). The specificity and sensitivity for SMRP to differentiate MPM from ADCA and BPE were 63.93% and 100.00%, respectively, for a cutoff of 24.05 nM (Table 5). Specificities and sensitivities were 80.33% and 72.50% with a cutoff of 1.61 ng/ml for CCL2, 67.21% and 82.50% with a cutoff of 14.60 ng/ml for LGALS3, and 70.49% and 80.00% with a cutoff of 168.20 ng/ml for SLPI (Table 5). Of interest, the SMRP/CCL2/galectin-3 marker combination reached an AUC of 0.968 (Fig. 4*B*) (Table 4).

DISCUSSION

MPM is a highly aggressive tumor associated with long-term asbestos exposure. The main challenge for this disease is its early and specific diagnosis. Accurate identification of MPM should improve the medical care of patients. Soluble markers seem to be interesting tools for rapid MPM diagnosis and for monitoring treatment response. In a previous study,

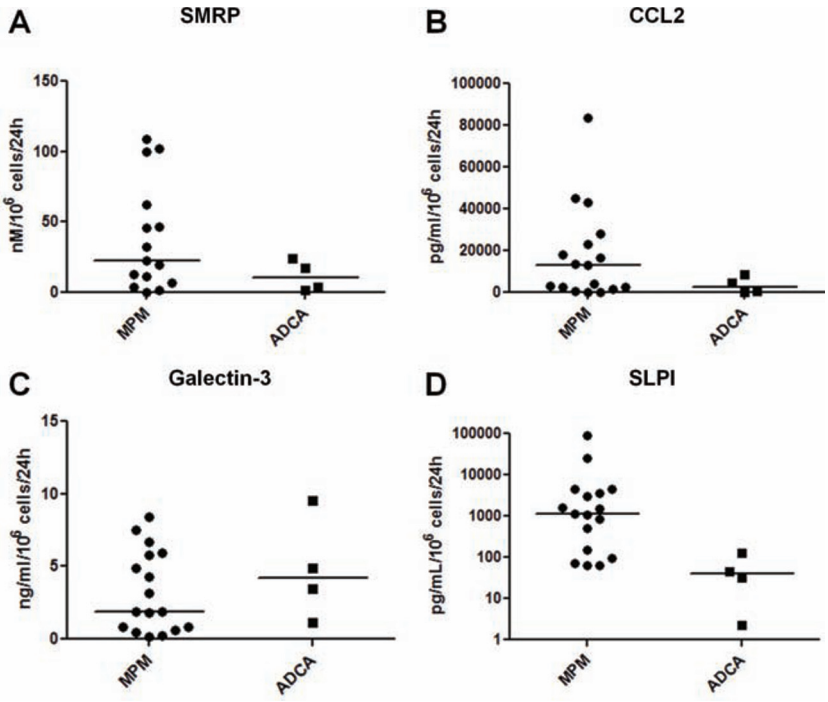

FIGURE 3. Measurements of SMRP, CCL2, galectin-3, and SLPI in cell culture supernatants. Culture supernatant SMRP (*A*), CCL2 (*B*), galectin-3 (*C*), and SLPI (*D*) values in MPM cells compared with ADCA cells. Horizontal lines, median values. SMRP, soluble mesothelin-related peptides; SLPI, secretory leukocyte peptidase inhibitor; MPM, malignant pleural mesothelioma; ADCA, adenocarcinoma.

we performed a transcriptomic study to compare MPM and pulmonary ADCA cell lines established from pleural fluids of patients.[17] From this work, we identified CCL2 and galectin-3 as new soluble markers to differentiate MPM from ADCA or BPE. We also found that SLPI mRNA was overexpressed in MPM cells. In this study, using commercially available ELISA assays we evaluated the levels of these biomarkers in pleural fluids collected over a 10-year period from patients with suspected MPM. As previously reported,[12,13] we found that in addition to SMRP, CCL2, galectin-3, and SLPI are good markers for differentiating MPM from ADCA and BPE in pleural fluids. Moreover, we have determined that an analytic

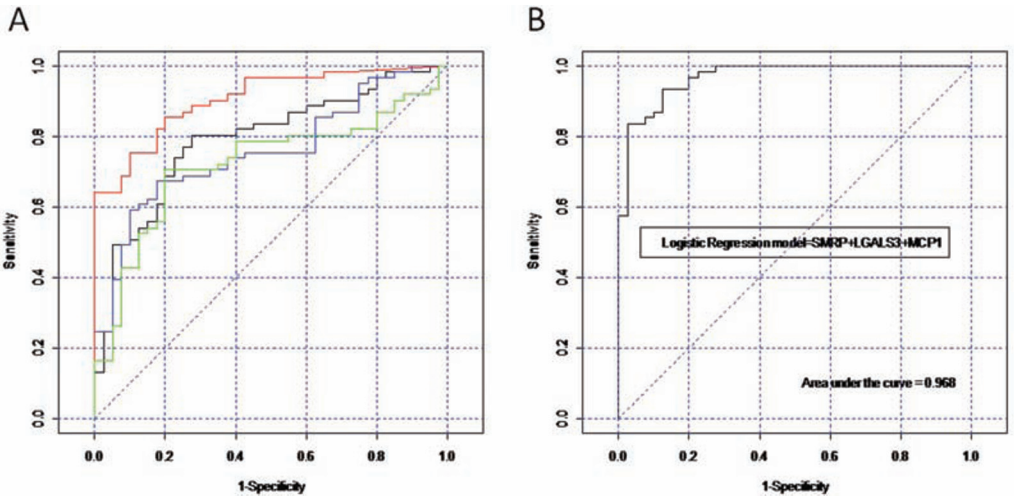

FIGURE 4. Diagnostic values of SMRP, SLPI, CCL2, and galectin-3, alone or in combination. (*A*) ROC curve for SMRP (red), SLPI (green), CCL2 (black) or galectin-3 (blue) to distinguish between patients with MPM and patients with ADCA or BPE. (*B*) ROC curve for the combination SMRP, CCL2, and galectin-3 to distinguish between patients with MPM and patients with ADCA or BPE. SMRP, soluble mesothelin-related peptides; SLPI, secretory leukocyte peptidase inhibitor; ROC, receiver operating characteristic; MPM, malignant pleural mesothelioma; ADCA, adenocarcinoma; BPE, benign pleural effusion.

TABLE 4. ROC Curve Data for Ability of Biomarkers to Differentiate MPM from ADCA and BPE

Marker	AUC	95% confidence interval	SE	p
SMRP	0.9059	0.8503–0.9616	0.02838	<0.0001
CCL2	0.7912	0.7020–0.8803	0.04547	<0.0001
LGALS3	0.7584	0.6652–0.8516	0.04752	<0.0001
SLPI	0.7219	0.6207–0.8231	0.05161	<0.001
SMRP,CCL2 and LGALS3	0.9680	0.9120–0.9930	0.0168	<0.0001

ROC, receiver operating characteristic; MPM, malignant pleural mesothelioma; ADCA, adenocarcinoma; BPE, benign pleural effusion; SMRP, soluble mesothelin-related peptides; LGALS3, galectin-3; SLPI, secretory leukocyte peptidase inhibitor.

TABLE 5. Best Cut-Off Values to Differentiate MPM from ADCA and BPE

Marker	Cut-off	Specificity %	Sensitivity %
SMRP	24.05 nM	63.93	100.00
CCL2	1.61 ng/ml	80.33	72.50
LGALS3	14.60 ng/ml	67.21	82.50
SLPI	168.20 ng/ml	70.49	80.00

MPM, malignant pleural mesothelioma; ADCA, adenocarcinoma; BPE, benign pleural diseases; SMRP, soluble mesothelin-related peptides; LGALS3, galectin-3; SLPI, secretory leukocyte peptidase inhibitor.

combination of SMRP, CCL2, and galectin-3 improves the differential diagnosis of MPM up to an AUC of 0.968, whereas SMRP alone is, in our use, 0.9059. This value is slightly higher than those reported by others, but in the same range.[12,13,18,19]

As previously observed, SMRP levels were higher in pleural fluids from patients with EM than in those from patients with SM or MM, ADCA or BPE.[12] The nondetection of SM by SMRP represented the main limitation of this marker.[12] Several attempts were made to improve MPM diagnosis by combining SMRP with biomarkers used for the diagnosis of other cancers, such as cytokeratin fragment, carcinoembryonic antigen, carbohydrate antigen 15-3, carbohydrate 125, and osteopontin. However, all of these combinations showed poor, or no, benefit compared with the biomarker alone.[20] We showed previously that chemokine CCL2 levels were higher in patients with MPM, whatever the subtype. However, when we performed a more detailed evaluation of the ADCA group, we found that the ability of CCL2 to discriminate MPM from pulmonary ADCA was not as good as expected. This suggested that a combination of SMRP and CCL2 allows the detection of MPM, including SM. However, this combination does not clearly distinguish MPM from pulmonary ADCA, which is unsatisfying for the diagnosis of MPM. Thus, the high levels of galectin-3 measured in the pleural fluids of pulmonary ADCA patients as compared with those of MPM and its subgroups justified its association with SMRP and CCL2. SLPI is an alarm antiprotease overexpressed in ovarian-cancer cells and associated with the promotion of malignancy.[21,22] Pleural fluid levels of SLPI were mainly elevated in epithelioid MPM patients, whereas SLPI levels were elevated in the other MPM subtypes, and the ADCA and BPE groups were similar, as observed for SMRP.

The results obtained from the experiments conducted with pleural liquids were reinforced by the analysis of culture supernatants from MPM and ADCA cell lines established from pleural fluids.[17] MPM cell lines produced higher levels of SMRP, CCL2, and SLPI than did ADCA cell lines. Conversely, ADCA cell lines produced higher levels of galectin-3 than did MPM cell lines. These data were in parallel with biomarker determinations in pleural fluids and confirmed their specificities.

The complementarities of these biomarkers were studied using multiple logistic regressions that showed CCL2 ($p = 0.002$), LGALS3 ($p = 0.035$), and SMRP ($p = 0.001$) as statistically significant biomarkers, whereas SLPI was not significant ($p = 0.28$) when adjusted with the other biomarkers, demonstrating its correlation with the other biomarkers. Moreover, ROC curve analysis using the combination SMRP/galectin-3/CCL2 (AUC = 0.968) showed an interesting improvement in MPM diagnosis as compared with SMRP alone (AUC = 0.9059). Using the SMRP/CCL2/galectin-3 combination to classify the pleural fluids, according to their best cutoff values, only seven of 106 samples were misclassified (one false positive and six false negatives). In all cases of false negatives, the reason for the misclassification of the sample was a high galectin-3 level. In two cases, the diagnoses were ambiguous and for two others, atypical cells were observed in the pleural fluid cytological examination. The false-positive sample was from a patient with rheumatoid polyarthritis presenting a bilateral pleurisy characterized by low levels of SMRP and galectin-3, and a high level of CCL2, probably resulting from his pathology.[23] Despite the existence of limits represented by these seven samples, the SMRP/CCL2/galectin-3 combination could clearly improve the diagnosis of MPM on the basis of the determination of their levels in pleural fluids. This combination should now be evaluated in multicenter studies to validate its potential utility for MPM diagnosis.

The determination of these new biomarkers in serum samples also represents an interesting option that avoids recourse to an invasive procedure, such as thoracoscopy. SMRP measurements in serum samples have previously been performed and shown diagnostic performance similar to those in pleural fluids.[12,13,24] Thus, at the least, CCL2 and galectin-3 determinations should be carried out on serum samples to evaluate their diagnostic value. As for SMRP, the correlation of CCL2 and galectin-3 levels in pleural fluids and in blood samples should be studied to determine whether these data are correlated or complementary.

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